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Increased Na pump activity in the kidney cortex of the Milan hypertensive rat strain

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The (Na^+,K^+)-ATPase activity from the kidney cortex of the Milan hypertensive rat strain (MHS) and the corresponding normotensive control (MNS) was measured both in active solubilized enzyme preparations and in isolated basolateral membrane vesicles. Kinetic analysis of the purified enzyme showed that the V_{max} value was significantly higher in MHS rats. The difference between MHS and MNS was not linked to a different number of sodium pumps, but was related to the molecular activity of the enzyme. Using basolateral membrane vesicles, an increased ATP-dependent ouabain-sensitive sodium transport was also demonstrated in MHS rats. These results support the hypothesis that a higher tubular sodium reabsorption may be involved in the pathogenesis of hypertension in this rat strain.

Sodium transport; (Na^+,K^+)-ATPase; Basolateral membrane vesicle; Kinetics; Hypertension; Rat kidney

1. INTRODUCTION

The Milan hypertensive rat strain (MHS) is a useful model for studying some types of renal hypertensive mechanisms that may be responsible for 'essential' hypertension in subgroups of human subjects [1]. MHS rats are characterized by a generalized, genetically transmitted defect in the structure and function of plasma membranes which affects the transport of several ions (for a Review see [21]). At kidney level, an abnormality in sodium handling with a transient cumulative sodium retention associated with the development of hypertension in MHS rats, has been described [3]. These results have been partially confirmed by in vitro biochemical techniques using purified plasma membrane preparations. In particular, an increased sodium transport across the luminal membranes of the proximal tubular cells [4,5] and an increase $Na^+/K^+/Cl^-$ cotransport activity in the thick ascending limb of Henle's loop has been demonstrated [6]. However, whether or not the primary defect in sodium transport is located at the apical membrane has not yet been confirmed. In fact, although microdissection studies performed on proximal tubular segments revealed an increased (Na^+,K^+)-ATPase activity in MHS rats [7], few data are available on sodium transport at the basolateral pole of kidney cells in MHS rats.

In this paper, some biochemical features of the (Na^+,K^+)-ATPase purified from the kidney cortex of MHS rats and the corresponding normotensive control (MNS) are presented. The results obtained with purified enzyme were also compared to the ATP-dependent sodium transport measured in purified basolateral membrane vesicles.

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2. MATERIALS AND METHODS

2.1. *Purification of the (Na^+,K^+)-ATPase*

Adult male MHS rats (body weight 200–220 g), and corresponding MNS control rats were used. For each preparation kidney cortex slices, obtained from 2–3 animals, were pooled and resuspended in the homogenization buffer 0.25 M sucrose, 0.25 M histidine, pH 7.2, 5 mM dithiothreitol (10 ml buffer/g tissue). The (Na^+,K^+)-ATPase was purified according to the procedure described by Jørgensen [8] as modified by Liang-Winter [9], using 0.33 mg sodium dodecyl sulfate (SDS)/mg of protein during the incubation of the microsomal fraction with the detergent. This ratio was chosen from the SDS-dependent activation curve of the (Na^+,K^+)-ATPase (Fig. 1).

2.2. *Enzyme assay*

The (Na^+,K^+)-ATPase activity was determined with a timed test tube assay in which released inorganic phosphate was quantified as described by Fiske and Subbarow [10]. The reaction mixture contained NaCl and KCl as specified in the individual experiments, 3 mM ATP, 3 mM $MgCl_2$, 1 mM EDTA, 30 mM Tris-Cl, pH 7.4, at 37°C and, unless otherwise stated, with or without 1 mM ouabain. The reaction was stopped after 15 min by the addition of 600 μl of ice-cold 0.6 N perchloric acid and centrifuged for 2 min in a Beckman Microfuge. For the organic phosphate assay 800 μl of the supernatant were used. The activities of the homogenate and crude extracts were determined after a preincubation of the sample for 20 min at room temperature with 0.33 mg SDS/mg of protein. Proteins were determined according to Bradford [11], using a Bio-Rad kit.

2.3. *Basolateral membrane vesicles preparation*

Basolateral membrane vesicles (BLMV) were prepared according to

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Boumendil-Podevin and Podevin [12]. For each preparation, kidney cortex slices obtained from 5 MHS and 5 MNS were used. Unless otherwise stated, BLMV were resuspended in 85 mM KCl, 85 mM sucrose and 10 mM HEPES-Tris, pH 7.4, at the protein concentration of 8–10 mg/ml. Membrane sidedness was determined as previously described [13], using 0.6 mg/ml gramicidin and 0.5 mg/ml SDS.

2.4. *Transport experiments*

Uptake of ^{22}Na into BLMV was measured by the rapid filtration technique as reported [4], with some minor changes. Briefly, incubations were started by mixing 5 μl of BLMV with 45 μl of a cocktail containing 220 mM sucrose, 2.3 mM $MgCl_2$, 8.8 mM NaCl, 4 mM ATP, 10 mM HEPES-Tris (pH 7.4), and 11.1 μCi/ml $^{22}NaCl$. At selected times, 50 μl samples were withdrawn from the incubation mixture, diluted in 5 ml of ice cold 150 mM $MgCl_2$, 10 mM HEPES-Tris, pH 7.4 (stop solution) and immediately filtered through a prewetted filter of cellulose nitrate (HAWP, Millipore, 0.45 μm pore size). The filter was washed twice with 5 ml stop solution, put into a vial and counted by means of a liquid scintillation spectrometer. The cpm values were transformed into pmols of sodium taken up by the vesicles and referred to mg of protein of the samples.

2.5. *^{3}H-ouabain binding studies*

Binding was performed in a 24-well automatic filtration apparatus (Brandel, Inc., Gaithersberg, MD). The enzyme was incubated for 60 min at 37°C in 50 mM Tris-Cl, pH 7.4, containing 100 mM NaCl, 3 mM $MgCl_2$ and different ^{3}H-ouabain concentrations.

2.6. *Calculations*

Kinetic parameters were calculated with an IBM personal computer using a non-linear regression analysis program (ENZFITTER, Elsevier-Biosoft).

2.7. *Materials*

Dithiothreitol and Na_2ATP were obtained from Boehringer (Mannheim, Germany); ouabain, digoxin, digitoxygenin and digitoxigenin were from Sigma (St. Louis, MO) and $^{22}NaCl$ (200 μCi/ml, carrier free) from Amersham (Amersham, UK). All other reagents were analytical grade products from Merck (Darmstadt, Germany) or Fluka (Bucks, Switzerland).

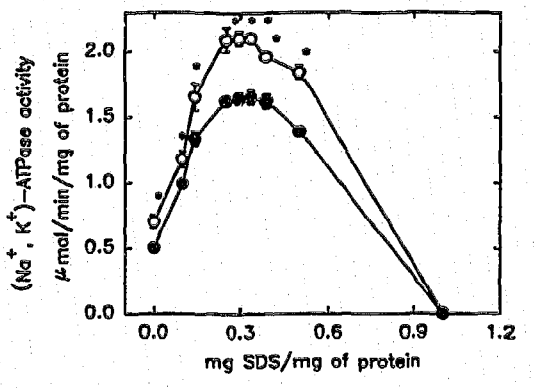

Fig. 1. Effect of SDS on (Na^+,K^+)-ATPase activity from kidney cortex of MHS and MNS rats. Enzyme activity was measured on the microsomal fraction of MHS (open symbols) and MNS (closed symbols) as described in section 2 and at the indicated SDS/protein ratio. Data obtained from 3 independent preparations were reported. Asterisks represent significant difference between MHS and MNS.

3. RESULTS

In Fig. 2A and B the kinetics of the (Na^+,K^+)-ATPase was studied at varying sodium and potassium concentrations. The initial rate of the sodium pump showed a sigmoidal saturation curve either when sodium or potassium was varied. The kinetic parameters are given in Table I. The difference between the two rat strains was evident at all sodium or potassium concentrations and was related to an increase in V_{max}, since K_{50} values for sodium and potassium and Hill coefficients were not statistically different between MHS and MNS rats. Binding studies with ^{3}H-ouabain confirmed that the increased V_{max} was not linked to a higher number of (Na^+,K^+)-ATPase molecules but to a faster catalytic

Table I

Kinetic parameters of the (Na^+,K^+)-ATPase from kidney cortex of MHS and MNS rats

Parameter	MNS	MHS	*P*
V_{max}	3274 ± 300 (11)	4629 ± 486 (11)	< 0.01
$K_{0.5}$ (Na^+)	20.9 ± 2.5 (4)	18.6 ± 1.9 (4)	NS
n (Na^+)	1.8 ± 0.3 (4)	2.2 ± 0.3 (4)	NS
$K_{0.5}$ (K^+)	3.1 ± 1.0 (7)	2.7 ± 0.7 (7)	NS
n (K^+)	2.1 ± 0.3 (7)	1.8 ± 0.2 (7)	NS

Values were calculated by best-fit analysis using the program ENZFITTER and an IBM Personal Computer. V_{max} is expressed in mU/mg protein, $K_{0.5}$ in mM and *n* is the Hill coefficient. Data represent the mean±SE of the number of experiments indicated in parentheses. Two-way analysis of variance has been used to evaluate the statistical significance between MHS and MNS preparations. NS=not significant.

Table II

Inhibition by ouabain and some ouabain-like compounds of sodium pump activity from kidney cortex of MHS and MNS rats

Compound	IC_{50}		
	MNS	MHS	*P*
Ouabain	69.5 ± 1.7 (3)	89.8 ± 17.7 (3)	NS
Digoxin	64.1 ± 10.8 (4)	60.6 ± 6.5 (4)	NS
Digitoxygenin	183.1 ± 17.0 (3)	232.5 ± 12.5 (3)	NS
Digitonin	134.0 ± 48.5 (3)	210.5 ± 29.6 (3)	NS

The (Na^+,K^+)-ATPase activity was determined in the presence of 10 mM KCl, 134 mM NaCl and at varying inhibitor concentrations (range 10^{-8} to 10^{-2} M). Values were calculated by best-fit analysis using the program ENZFITTER and an IBM Personal Computer. IC_{50} is in μM±SE of the number of experiments reported in parentheses. Two-way analysis of variance has been used to evaluate the statistical significance between MHS and MNS preparations. NS=not significant.

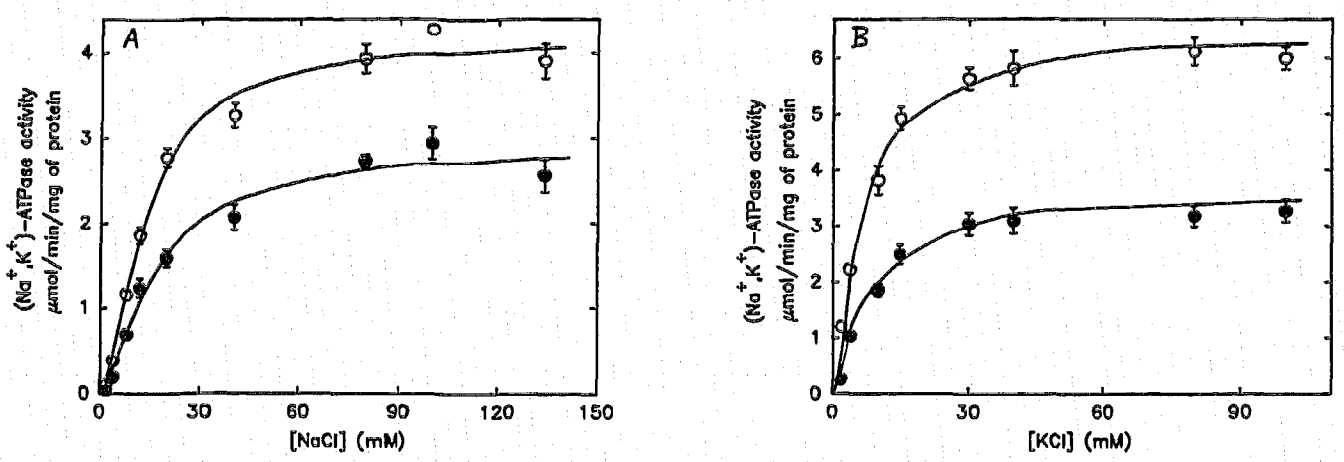

Fig. 2. Effect of varying sodium and potassium concentration on (Na^+,K^+)-ATPase activity from kidney cortex of MHS and MNS rats. Enzyme activity was measured as described in section 2 in the presence of 10 mM KCl, and NaCl concentrations ranging from 2 to 134 mM (panel A) or in the presence of 134 mM NaCl, and KCl concentrations varying from 2 to 100 mM (panel B). Open symbols = MHS; closed symbols = MNS. The data represent the means of at least 4 independent preparations.

rate of the enzyme. As a matter of fact, the number of ouabain binding sites was 2.9±0.3 and 2.6±0.2 nmol/mg of protein for MHS and MNS, respectively. From these data and the enzyme activity reported in Table II, the turnover numbers 26.6 s^{-1} and 20.9 s^{-1} for MHS and MNS, respectively, were calculated.

To further characterize the enzyme preparation, the sensitivity of the purified (Na^+/K^+)-ATPase to some digitalic compounds was examined and dose–response curves for ouabain, digoxin, digitonin and digitoxygenin were established for each of them. The calculated IC_{50} are listed in Table II. The order of potency of the compounds tested was not different in the two rat strains, being digoxin ≈ ouabain > digitonin ≈ digitoxygenin. The same inhibition pattern for digitalics seems to indicate that the binding site for ouabain-like compounds has a quite similar structure in the two rat strain.

In order to verify that differences between MHS and MNS in (Na^+,K^+)-ATPase activity represented differences in sodium transport across the basolateral membrane of proximal tubular cells, ATP-dependent sodium transport in BLMV isolated from kidney cortex of the two rat strains was measured. Since sodium uptake in BLMV through the sodium pump depends on the accessibility of the ATP binding site, membrane sidedness has to be measured if difference between two membrane preparations must be compared. As reported in Table III, the percentage of leaky, inside-out and rightside-out vesicles were not significantly different between MHS and MNS rats. As shown in Fig. 3, addition of ATP in the presence of extravesicular Mg^{2+} to K^+-loaded BLMV stimulated sodium uptake both in MHS and in MNS. However, the ATP-dependent component of the total sodium uptake, after correction for the proportion of inside-out vesicles present in each preparation, was significantly higher in MHS than in MNS rats, confirming that an increased sodium pump activity was present at the basolateral pole of the proximal tubular cells in hypertensive rats. Table IV further demonstrated that the ATP-stimulated sodium transport is ouabain-inhibitable and can therefore be ascribed to the activity of the (Na^+,K^+)-ATPase. The ouabain-sensitive

Table III

Sidedness of BLMV from kidney cortex of MHS and MNS rats

Vesicles	% of total		
	MNS	MHS	*P*
Leaky	48 ± 3	56 ± 4	NS
Right-side out	35 ± 2	32 ± 2	NS
In-side out	17 ± 3	12 ± 2	NS

Leaky vesicles are calculated from the ratio: (Na^+,K^+)-ATPase activity without SDS/(Na^+,K^+)-ATPase activity with 0.5 mg/ml SDS; right-side-out vesicles are: 1 – (total ATPase activity without SDS/total ATPase activity with 0.5 mg/ml SDS). Values represent the mean±SE of 4 independent preparations. Two-way analysis of variance has been used to evaluate the statistical significance between MHS and MNS preparations. NS=not significant.

Table IV

Effect of ouabain on ATP-dependent sodium transport in BLMV from MHS and MNS rats

Condition	MNS		MHS	
	−ouabain	+ouabain	−ouabain	+ouabain
+ATP	176 ± 15*	106 ± 8	247 ± 14*	134 ± 7
−ATP	62 ± 10	53 ± 5	67 ± 8	55 ± 10

Sodium uptake was determined as described in Fig. 3 after pre-loading the vesicles with or without 2 mM ouabain. The values are expressed in nmol/10 s/mg of protein ±SE of 3 independent experiments. Asterisks represent significant difference between MHS and MNS.

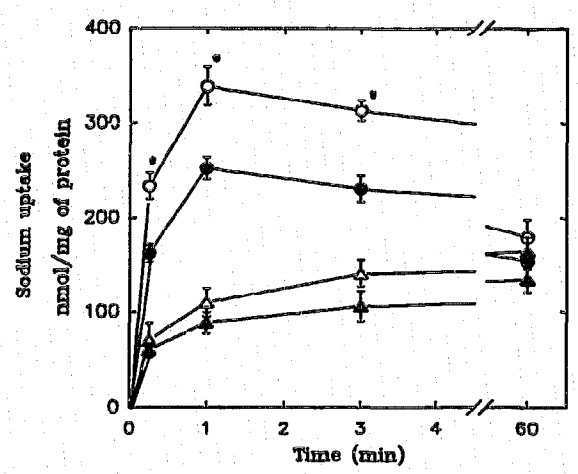

Fig. 3. Sodium transport across BLMV isolated from kidney cortex of MHS and MNS rats. Sodium uptake in BLMV from MHS (open symbols) and MNS (closed symbols) was measured as described in section 2 in the presence (circles) or in the absence (triangles) of 4 mM ATP. Asterisks represent significant differences between MHS and MNS.

sodium uptake was 70±11 and 113±12 nmol/10 s/mg protein for MNS and MHS, respectively (n=4, P<0.05, t-test). By contrast, the ATP-independent sodium uptake was unaffected by ouabain and not significantly different between the two rat strains. The presence of an ouabain-insensitive ATP-dependent sodium uptake (Table IV, column 2 vs 4) is unlikely to be due to another ATP- dependent sodium transport, i.e. the Na^+-ATPase [14], because a sodium-stimulated furosemide-sensitive ATP splitting was undetectable in our membrane preparations. The discrepancy may be explained supposing that ouabain does not reach effectively all intravesicular binding sites during the preloading procedure.

4. DISCUSSION

A higher (Na^+,K^+)-ATPase specific activity in kidney cortex from MHS rats has been demonstrated both in active solubilized enzyme preparations and purified BLMV. The different V_{max} values were independent of the purification procedure. In fact, the recovery of the (Na^+,K^+)-ATPase activity from kidney cortex of MHS and MNS rats was the same in the two strains. The enrichment factors between homogenate and the final pellet of the solubilized enzyme were 22.2±2.2 and 21.9±2.0 for MHS and MNS, respectively. All the ATPase activity was (Na^+,K^+)-dependent, since it was completely abolished by the presence of 2.5 mM ouabain. Moreover, the obtained data cannot be ascribed to different unmasking effects of SDS, because the shape of the SDS-activation curve was the same in the two rat strains and a significant difference was present also in the absence of detergent (Fig. 1). Such an increased activity seems to be due to a higher turnover number, as supported by ^{3}H-ouabain binding experiments and by recent data obtained by in situ hybridization techniques showing no difference in the number of α isoforms of (Na^+,K^+)-ATPase in proximal tubules of MHS and MNS rats (T. Cova and V. Herrera, personal communication).

The experiments performed on membrane vesicles revealed that in MHS the ^{22}Na uptake was significantly higher than in MNS. The observed differences were not linked to the degree of contamination by non-basolateral membranes (no differences between MHS and MNS, data not shown) or to a different membrane sidedness (Table III), but can be ascribed to an increased activity of the sodium pump in MHS rats.

The data reported in the present paper confirm previous results obtained on proximal convolute tubule segments [7], in which a higher (Na^+,K^+)-ATPase activity per mm of tubule and a faster ouabain-sensitive O_2 consumption have been demonstrated in MHS rats. An increase in the sodium pump activity in the proximal tubular cells of MHS rats is in agreement with the increase in sodium transport across the brush border membrane [4,5] and with the lower intracellular sodium content compared to the normotensive control [15]. Moreover, previous experiments on isolated perfused kidney of MHS and MNS showed that addition of 1 mM ouabain to the perfusate induced a greater natriuretic response in MHS kidney, in keeping with a greater ouabain-sensitive Na^+ reabsorption [16]. Taken together, these results fit with the physiological data available on MHS rats, among which the most relevant are a higher whole-kidney glomerular filtration rate (GFR) [1,17], a lower kidney weight [1,17], the ability to maintain a normal sodium balance despite the faster GFR [18] and a blunted tubulo-glomerular feedback mechanism [19]. In fact all these data can be explained by a primary increase of proximal tubular reabsorption. Therefore, several physiological and biochemical data seem to indicate that some alterations in membrane transport of sodium, both at the luminal and basolateral pole, are present in the proximal tubules of MHS rats. Which is the pathway that primarily drives the faster sodium reabsorption or, in other words, which is the molecular defect responsible for these membrane abnormalities remains to be elucidated.

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